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Regulation of cytochrome P-450b/e gene expression by a heme- and phenobarbitone-modulated transcription factor

(gel retardation/footprinting/protein blot)

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ABSTRACT **The cloned DNA fragment of the cytochrome P-450b/e gene containing the upstream region from position −179 through part of the first exon is faithfully transcribed in freeze–thawed rat liver nuclei. Phenobarbitone treatment of the animal strikingly increases this transcription, and the increase is blocked by cycloheximide (protein synthesis inhibitor) or $CoCl_2$ (heme biosynthetic inhibitor) treatment of animals. This picture correlates very well with the reported cytochrome P-450b/e mRNA levels *in vivo* and run-on transcription rates *in vitro* under these conditions. The upstream region (from position −179) was assessed for protein binding with nuclear extracts by nitrocellulose filter binding, gel retardation, DNase I treatment ("footprinting"), and Western blot analysis. Phenobarbitone treatment dramatically increases protein binding to the upstream region, an increase once again blocked by cycloheximide or $CoCl_2$ treatments. Addition of heme *in vitro* to heme-deficient nuclei and nuclear extracts restores the induced levels of transcription and protein binding to the upstream fragment, respectively. Thus, drug-mediated synthesis and heme-modulated binding of a transcription factor(s) appear involved in the transcriptional activation of the cytochrome P-450b/e genes, and an 85-kDa protein may be a major factor in this regard.**

Interaction of specific transcription factors with the cis-acting elements in the 5′-flanking region of several eukaryotic genes governs ligand-, tissue-, and cell-specific modulation of gene expression. Thus, families of transcription factors interacting with specific regulatory DNA sequences of eukaryotic genes have been identified (1).

The cytochrome P-450 (P-450) multigene family offers an excellent system to study regulation of eukaryotic gene expression, because this family responds to a variety of environmental and developmental stimuli (2). Various reports identify cis-acting elements, referred to as dioxin-responsive elements, in different locations ranging from positions −389 to −2263 of the mouse P-450 gene activated by polycyclic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and 3-methylcholanthrene (3-MC) (3, 4). Xenobiotic responsive elements have been identified ≈1.0 kilobase (kb) upstream of the RNA initiation site of the hydrocarbon-responsive P-450c gene of rat liver and a xenobiotic responsive element-binding factor that is translocated from the cytoplasm into the nucleus after dioxin treatment has also been identified (5, 6). No information is available on the function of the upstream regulatory elements of the P-450b/e genes (phenobarbitone family).

In this laboratory, we have provided evidence that heme, the prosthetic group of cytochrome P-450, is a positive modulator of P-450 gene expression. The evidence is as follows: (*i*) Inhibitors of heme biosynthesis block induction of P-450b/e and P-450c/d mRNAs by phenobarbitone and 3-MC, respectively, in rats. (*ii*) Effects of these inhibitors can be counteracted by exogenous hemin. (*iii*) Changes in the mRNA levels *in vivo* correlate with their run-on transcription rates with nuclei isolated from treated rat livers (7–10). In addition, we have also shown that cycloheximide, a translation inhibitor, specifically blocks the transcriptional activation of P-450b/e and -c/d genes by phenobarbitone and 3-MC, respectively (11).

We reported the 5′-flanking sequence of the P-450b/e gene to position −800, a sequence that contains several potential regulatory sites (12). In the present study, we identify a drug- and heme-modulated transcription factor(s) from rat liver. Binding of this factor(s) to the 5′-flanking region of the P-450b/e gene correlates well with the transcription status of these genes.

MATERIALS AND METHODS

DNA Fragments and Probes. The plasmid pP450e4 was used in *in vitro* transcription experiments. This plasmid contains a 360-base pair (bp) fragment of the P-450e gene (identical with the P450b gene except for four base substitutions) cloned into the *Sma* I site of plasmid pUC19. The fragment contains nucleotides from position −179 of the upstream region and 181 nucleotides (nt) of the transcribed region. The 360-bp fragment released with *Sal* I and *Eco*RI (polylinker sites) from pP450e4 has a unique *Nco* I site at position 29. The transcripts obtained in the *in vitro* transcription experiments were analyzed by S1 nuclease protection assay with a *Nco* I–*Eco*RI fragment (170 bp) that covered some of the first exon labeled at the *Nco* I end and representing the coding strand (Fig. 1).

For nitrocellulose filter binding assays, the 360-bp fragment released by *Eco*RI and *Sal* I and labeled at the 3′ end with [α-^{32}P]dCTP was used. Gel-retardation and DNase I protection "footprinting" assays were performed with the *Sal* I–*Nco* I fragment (223 bp), which covered the upstream region and 29 bp of the first exon. The DNA sequence, restriction sites, and orientation of the probes appear in Fig. 1.

***In Vitro* Transcription and S1 Nuclease Protection Assays.** Cloned DNA, pP450e4, was added to freeze–thawed nuclei and incubated under conditions described by Guertin *et al.* (13). No radioactive precursor was added. The RNA transcripts were isolated after DNase I and proteinase K treatments and then hybridized to labeled *Nco* I–*Eco*RI fragment (50,000 cpm), and S1 nuclease protection analysis was done as described by Favaloro *et al.* (14). The protected fragment was analyzed on urea–acrylamide (8%) gels and subjected to autoradiography.

Nitrocellulose Filter Binding Assay. Nuclei were prepared by citric acid buffer homogenization (15), the nuclear pellet

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Abbreviations: 3-MC, 3-methylcholanthrene; P-450, cytochrome P-450; nt, nucleotide(s).

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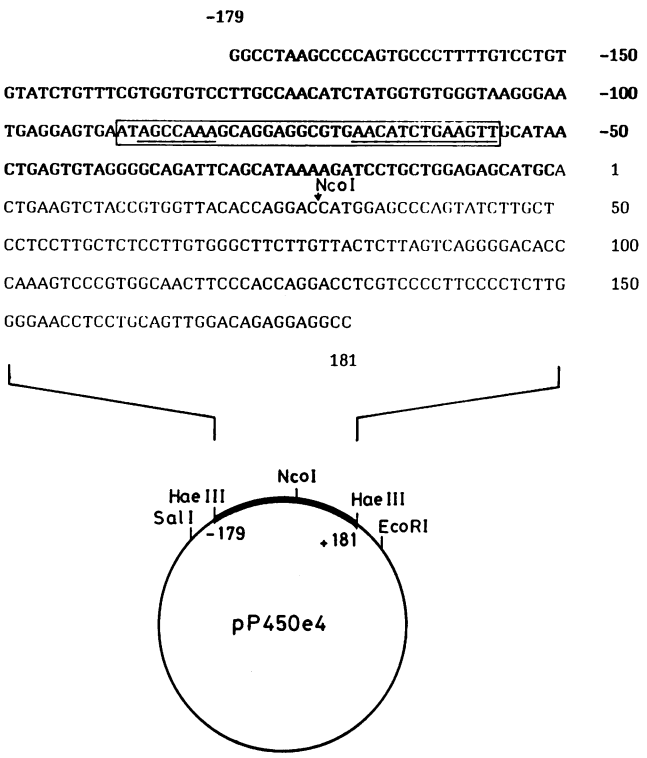

FIG. 1. Nucleotide sequence of clone pP450e4. This plasmid contains the 360-bp *Hae* III fragment of the P-450e gene (identical with P-450b gene, except for four base substitutions) cloned into the *Sma* I site of pUC19. The upstream region (−179) from the RNA start site is represented in boldface letters, and the exonic region is represented in regular letters. The fragment was obtained by *Hae* III digestion of the 1.0-kilobase (kb) fragment derived from clone λP-450e5 (12). The 1.0-kb fragment contains the nt from position −800 of the upstream region and 228 nt downstream. Polylinker sites and the unique *Nco* I site used to generate the probes are indicated; DNase I footprint region is boxed; consensus CCAAT binding site and 13-bp imperfect palindrome are underlined.

was extracted, and the extract was dialyzed as described by Strauss and Varshavsky (16). Filter binding assay was carried out essentially as described by Diffley and Stillman (17). Nuclear extracts were prepared by the addition of NaCl to the nuclear suspension (suspension buffer was 10 mM Tris·HCl, pH 7.5/50 mM NaCl/0.5 M sucrose/5 mM $MgCl_2$/10 mM dithiothreitol) to a final concentration of 0.45 M from a 4 M stock solution. The suspension was left on ice for 30 min with intermittent mixing; the suspension was then centrifuged, and the supernatant was dialyzed against 10 mM Tris·HCl, pH 7.5/1 mM EDTA/15% glycerol/10 mM 2-mercaptoethanol for 4 hr. The dialyzate was briefly centrifuged and stored in aliquots at −80°C. The nuclear extract (3–5 μg of protein) was then incubated with different concentrations of competitor DNA (sonicated salmon sperm or calf thymus DNA) in a volume of 20 μl in a buffer containing 25 mM Hepes·NaOH, pH 7.5, 5 mM $MgCl_2$, 2 mM dithiothreitol, and 50 mM NaCl at room temperature for 10 min. ^{32}P-end-labeled 360-bp *Sal* I–*Eco*RI fragment (3000 cpm, ≈0.5 ng of DNA) was then added, and incubation was continued for another 30 min. The mixture was then filtered through alkali-washed nitrocellulose filters and washed three times—each time with 0.5 ml of wash buffer (25 mM Tris·HCl, pH 7.5/5 mM $MgCl_2$). The filters were then dried and subjected to autoradiography.

Gel-Retardation Assay. The method was basically as described by Ye and Samuels (18). For assay of the interaction between nuclear extract and ^{32}P-end-labeled pP450e4 fragment, sonicated salmon sperm DNA was added at 400 ng. After incubation, 2 μl of loading buffer [0.05% bromophenol blue in 50% (vol/vol) glycerol] was added, and the samples were analyzed on a 4% polyacrylamide gel (acrylamide/bisacrylamide 30:1) in low-ionic-strength buffer (6.7 mM Tris·HCl, pH 7.5/3.3 mM sodium acetate/1 mM EDTA) at 4°C for 3 hr (10 v/cm). The gel was then soaked in 10% glycerol, dried, and subjected to autoradiography.

DNase I Footprinting. This procedure was carried out essentially as described by Galas and Schmitz (19). DNA sequencing was done by the method of Maxam and Gilbert (20). The conditions were the same as for gel-retardation analysis with the ^{32}P-labeled *Nco* I–*Sal* I fragment, except that the reaction was scaled up. DNase I was added to a final concentration of 0.3 μg/ml and digested for 60 sec at room temperature. The samples were extracted with phenol/chloroform, and DNA fragments were precipitated with ethanol. Fragments were analyzed on urea–polyacrylamide (8%) sequencing gels.

Western Blotting. Nuclear extracts (100–150 μg of protein) were subjected to $NaDodSO_4$/8% PAGE and transferred to nitrocellulose by electrophoresis. Samples were mixed with an equal volume of loading buffer (4% $NaDodSO_4$/5 mM Tris·HCl, pH 6.8/200 mM 2-mercaptoethanol/20% glycerol/0.05% bromophenol blue). The boiling step was omitted. After electrophoresis, proteins were transferred to nitrocellulose membrane by electrophoresis in a buffer containing 25 mM Trizma base and 190 mM glycine for 16 hr at 100 mA. The filters were first incubated in a blocking buffer (2% nonfat dry milk/1% bovine serum albumin/10 mM Hepes·NaOH, pH 7.5/0.1 mM EDTA/60 mM NaCl/10 mM $MgCl_2$/sonicated salmon sperm DNA at 16 μg/ml) for 2 hr at room temperature. The filters were then incubated in binding buffer (same as blocking buffer except for 0.2% nonfat dry milk) containing nick-translated *Sal* I–*Eco*RI 360-bp pP450e4 DNA fragment (10^6 Cerenkov cpm per ml) for 2 hr at room temperature. The filters were then washed four times with wash buffer (10 mM Tris·HCl, pH 7.5/0.1 mM EDTA/60 mM NaCl/0.05% Nonidet P-40/0.2% nonfat dry milk) at room temperature, dried, and subjected to autoradiography. The basic protocols were as described by Miskiminis *et al.* (21).

RESULTS

In most protein coding genes, sequences up to 100 bp upstream from the RNA start site regulate transcription, although complex regulation is achieved by additional sequences that may be located even as far as 10,000 bp upstream or downstream of the RNA start site (22). To examine whether the near-upstream sequence regulates P-450b/e gene expression, the plasmid pP450e4 containing base pairs from position −179 upstream from the RNA start site through a portion of the first exon (Fig. 1) was transcribed in freeze–thawed nuclei isolated from rat livers under different treatments. The transcripts were analyzed by S1 nuclease mapping using labeled *Nco* I–*Eco*RI fragment derived from the first exon. This treatment protected the 170- and 152-bp fragments by transcripts derived from the added cloned DNA and endogenous P-450b/e genes, respectively. (The strategy is explained in *Materials and Methods* and in Fig. 2*a*). The results (Fig. 2*b*) clearly indicate that the added cloned DNA is transcribed in nuclei isolated from phenobarbitone-treated rats several-fold higher than in control nuclei.

Nuclei isolated from heme-deficient rats (phenobarbitone plus $CoCl_2$-treated) show a striking decrease in transcription of the added gene fragment; addition of hemin *in vitro* counteracts the effects of $CoCl_2$ treatment. Without added DNA, the endogenous P-450b/e gene is significantly transcribed in nuclei isolated from phenobarbitone-treated rats, producing the expected 152-bp fragment. With added cloned DNA, transcription of endogenous P-450b/e gene is suppressed. Thus, freeze–thawed nuclei can faithfully transcribe

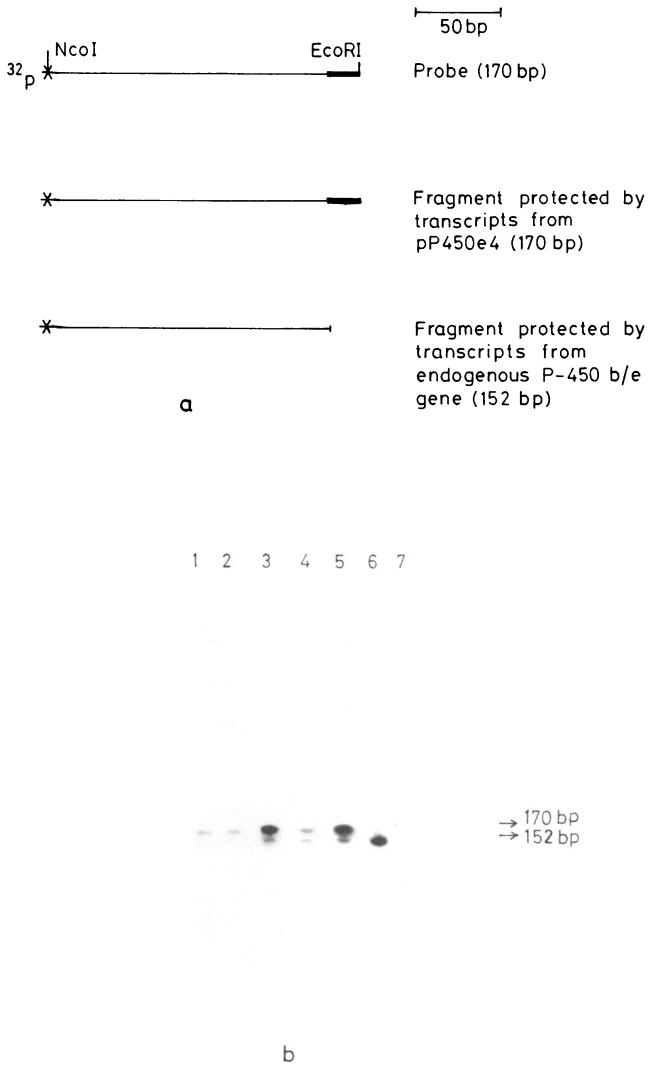

FIG. 2. Transcription of pP450e4 DNA with frozen–thawed rat liver nuclei *in vitro*. (*a*) The probe used for S1 nuclease protection analysis of the *in vitro* transcripts and the generation of different-sized protected fragments derived from endogenous transcripts and protected fragments derived from added pP450e4 plasmid are indicated. Thick line (18 bp) indicates the polylinker region. (*b*) Autoradiographic analysis of S1 nuclease-protected fragments generated by the RNA transcripts. Lanes: 1, no RNA; 2, control nuclei and pP450e4 DNA; 3, phenobarbitone nuclei and pP450e4 DNA; 4, phenobarbitone plus $CoCl_2$ nuclei and pP450e4 DNA; 5, phenobarbitone plus $CoCl_2$ nuclei and pP450e4 DNA plus hemin (10^{-5} M) added *in vitro*; 6, phenobarbitone nuclei with no added DNA; 7, phenobarbitone nuclei and pUC19 DNA. Animals were sacrificed 4 hr after drug and other treatments.

added P-450b/e gene fragment, and the result correlates well with changes in P-450b/e mRNA levels *in vivo* and run-on transcription rates *in vitro* reported earlier (8, 10). Because transcription of the added gene fragment should involve fresh initiation, the upstream sequence (from position −179) probably has sites for interaction with protein factors modulating transcription of P450b/e genes.

Therefore, nitrocellulose filter binding was assayed with nuclear extracts and labeled *Sal* I–*Eco*RI 360-bp fragment containing the upstream sequence and part of the first exon. The results (Fig. 3) indicate that when binding is done with 400 ng of unlabeled competitor DNA and ^{32}P-labeled 360-bp fragment, the nuclear extracts prepared from phenobarbitone-treated rat liver (phenobarbitone-nuclear extract) bind significantly, whereas the control extract shows little binding. Treatment of animals with cycloheximide or $CoCl_2$

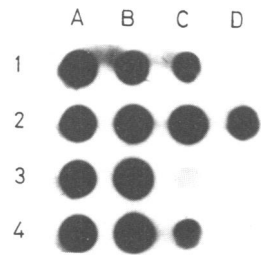

FIG. 3. Nitrocellulose filter binding assay of the interaction between the nuclear extracts and the labeled pP450e4 fragment. Rows: 1, control extract; 2, phenobarbitone extract; 3, phenobarbitone plus cycloheximide extract; 4, phenobarbitone plus $CoCl_2$ extract. Columns: A, no competitor DNA; B, 200 ng; C, 300 ng; D, 400 ng of competitor DNA.

blocks the significant binding seen with the phenobarbitone-nuclear extracts. These results are significant because they correlate exactly with the pattern of P-450b/e gene transcription obtained under the different treatment conditions. The results obtained with the nitrocellulose filter binding assay have been rather variable in quantitative terms, although the pattern obtained has been highly reproducible.

Confirmation of these results was obtained with gel-retardation experiments. For this purpose, the 360-bp *Sal* I–*Eco*RI fragment or the *Nco* I-cut fragments *Sal* I–*Nco* I and *Nco* I–*Eco*RI were used. Fig. 4 *a* and *b* shows that the major complex seen with phenobarbitone-nuclear extract is barely visible in control nuclear extract. The inhibitory effects of $CoCl_2$ and cycloheximide treatments are also clearly seen. Interestingly addition of hemin *in vitro* to heme-deficient nuclear extracts restores complex formation (Fig. 4*b*). In separate experiments, we also found that nuclear extracts prepared from phenobarbitone plus $CoCl_2$-treated animals given hemin *in vivo* also significantly restore (≈60%) complex formation (data not shown). Fig. 5*a* indicates that protein binds mostly with the *Sal* I–*Nco* I fragment (covering from position −179 of the upstream region to position 29 of the exon) and much less with the *Nco* I–*Eco*RI fragment (covering some of the first exon). This result shows binding to occur essentially with the upstream region. Fig. 5*b* indicates specificity of binding; binding is abolished with excess un-

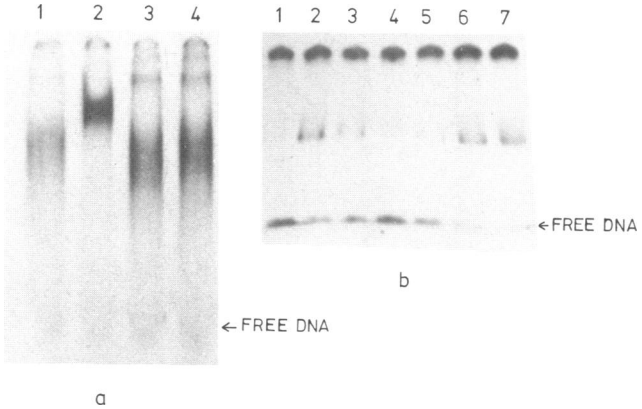

FIG. 4. Gel-retardation assay of the interaction between the nuclear extract and ^{32}P-end-labeled pP450e4 fragment. (*a*) Lanes: 1, control extract; 2, phenobarbitone extract; 3, phenobarbitone plus cycloheximide extract; 4, phenobarbitone plus $CoCl_2$ extract; *Sal* I–*Eco*RI (360 bp) fragment was used. (*b*) Lanes: 1, control extract; 2, phenobarbitone extract (1-hr treatment); 3, phenobarbitone and $CoCl_2$ extract (1-hr treatment); 4, phenobarbitone and $CoCl_2$ extract (3-hr treatment); 5, phenobarbitone and $CoCl_2$ extract (5-hr treatment); 6, phenobarbitone and $CoCl_2$ extract (5-hr treatment) plus hemin (10^{-5} M) *in vitro*; 7, phenobarbitone and $CoCl_2$ extract (5-hr treatment) plus hemin (10^{-6} M) *in vitro*. *Sal* I–*Nco* I (223-bp) fragment was used. Incubation conditions were as described for Fig. 3.

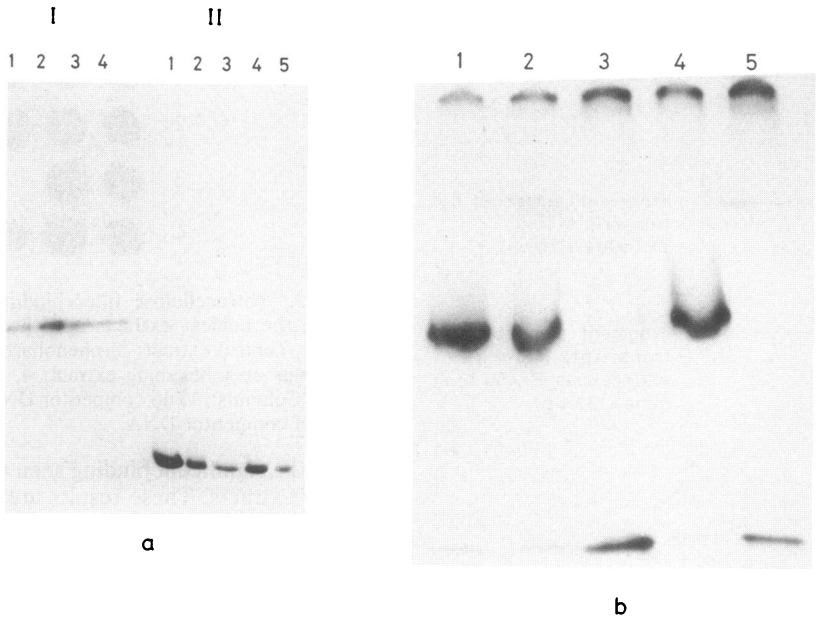

FIG. 5. Gel-retardation assay. (*aI*) *Sal* I–*Nco* I (223 bp) upstream fragment was used as a probe. Lanes: 1, control extract; 2, phenobarbitone extract; 3, phenobarbitone and $CoCl_2$ extract; 4, phenobarbitone and cycloheximide extract. (*aII*) *Nco* I–*Eco*RI (170 bp) exon fragment was used as probe. Lanes: 1, free DNA; 2, control extract; 3, phenobarbitone extract; 4, phenobarbitone and $CoCl_2$ extract; 5, phenobarbitone and cycloheximide extract. (*b*) Labeled *Sal* I–*Nco* I upstream fragment was used with phenobarbitone extract and different combinations of competitor DNA. Lanes: 1, 300 ng of salmon sperm DNA plus 100 ng of pUC19 DNA; 2, 300 ng of salmon sperm DNA plus 100 ng of M13 DNA; 3, 300 ng of salmon sperm DNA plus 100 ng of unlabeled *Sal* I–*Nco* I fragment; 4, 400 ng of salmon sperm DNA; 5, free labeled DNA.

labeled homologous fragment but not with excess pUC19 or M13 DNA fragments.

Fig. 6 shows the DNase I footprint obtained with the phenobarbitone-nuclear extract and the *Sal* I–*Nco* I fragment. A 32-bp protected region between nt −56 and −88 can be discerned.

Finally, Western blot analysis of the nuclear extracts was done, and the blots were probed with nick-translated pP450e4 DNA. The results (Fig. 7) show a striking increase in the band corresponding to M_r 85,000 in phenobarbitone treatment, which is abolished with $CoCl_2$ or cycloheximide treatments. A few other bands also increase to a minor degree after phenobarbitone treatment. The intense bands seen at the bottom of the gel are generated by histones.

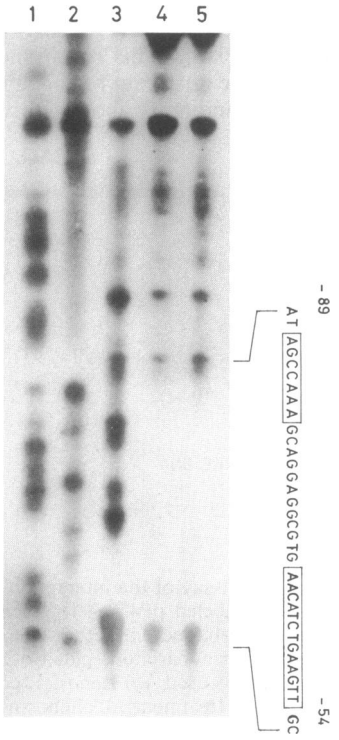

FIG. 6. DNase I footprinting analysis. Lanes: 1, G reaction; 2, C reaction; 3, without nuclear extract; 4, phenobarbitone-nuclear extract (50 μg of protein); and 5, phenobarbitone-nuclear extract (75 μg of protein). Protected sequence is indicated.

DISCUSSION

This study has led to several important observations. The cloned P-450b/e gene fragment containing base pairs from position −179 of the upstream region and some of the first exon is faithfully transcribed in freeze–thawed nuclei, exactly reflecting the drug-mediated induction, as well as the block seen under heme-deficient conditions in mRNA levels *in vivo* and run-on transcription experiments *in vitro*.

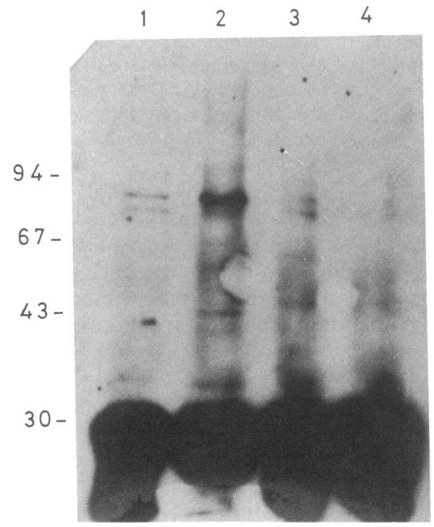

FIG. 7. Western blot analysis. Lanes: 1, control extract; 2, phenobarbitone extract; 3, phenobarbitone plus $CoCl_2$ extract; and 4, phenobarbitone plus cycloheximide extract.

Nuclear protein-binding experiments revealed that binding of a protein factor(s) to the upstream region significantly increases after phenobarbitone treatment, and this increase is blocked by heme deficiency or cycloheximide treatment. This result has been confirmed with nitrocellulose filter binding, gel retardation, and Western blot analysis. The striking correlation between patterns of binding and transcription under different treatment conditions indicates that the drug may induce synthesis of a transcription factor, which itself is hemoprotein in nature; binding of this factor to the upstream region would activate transcription. Cycloheximide would then block P-450b/e gene transcription because it blocks synthesis of the transcription factor. $CoCl_2$ can block transcription because $CoCl_2$ inhibits heme synthesis and the hemoprotein transcription factor requires heme for binding. This suggestion is supported by the observation that binding of such extracts to the DNA fragment is restored by adding hemin *in vitro*. Other models can be based on heme regulation of the phosphorylation status of the transcription factor and modulation of association/dissociation with an inhibitor. Further studies are needed to distinguish between these various hypotheses.

This report provides direct evidence for the involvement of heme in modulating interaction of the promoter region with a transcription factor(s); heme has earlier been reported to regulate the transcription of iso-1-cytochrome *c* gene in yeast (23). A heme-activated protein (*HAP1* gene product) was suggested to pick up heme in the cytosol and bind to the upstream activation sequence of the iso-1-cytochrome *c* gene. The *in vitro* addition of heme was shown to stimulate *HAP1* binding to the upstream activation sequence. The similarity of these observations to the present study is striking.

Recently, *HAP* gene products (HAP2 and HAP3 proteins) along with a host of other eukaryotic transcription factors have been categorized as CCAAT binding proteins. These proteins show similarities in their preferences for different forms of their DNA-binding sites, making identical contacts with the bound DNA. Such factors thus show specificity, while at the same time performing diverse functions, such as activation of transcription and replication—perhaps through microheterogeneity in their structures and heteromer formation (24, 25). An analysis of the 32-bp protected fragment obtained in our study does indicate the presence of the NGCCAAN sequence, the consensus sequence for nuclear factor I and other factors (24), followed by a 13-bp imperfect palindrome (Fig. 1).

At this stage, whether the transcription factor(s) identified in our study can be a drug receptor as well is not clear. Although the hydrocarbons act through a receptor (2), efforts to identify a receptor protein for phenobarbitone have not been very successful (26). Also the question remains whether the same or a similar transcription factor is induced by 3-MC because we have shown that both cycloheximide and $CoCl_2$ block 3-MC-mediated activation of P-450c/d genes as well (11). Further studies are needed to resolve many of these issues. Clearly, however, drug-mediated synthesis and heme-modulated binding of specific transcription factor(s) are involved in transcriptional activation of the P-450b/e gene system, and an 85-kDa protein may play a major role in this activity.

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